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REPORT NUMBER 14

Malaria: Biology of the Merozoite-Erythrocyte Interface

FINAL REPORT

Robert O. McAlister, Ph.D.

July 1978

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-75-C-5037

Southern Methodist University
Dallas, Texas 75275

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Studies were performed to develop a method for the isolation of large numbers of free merozoites of Plasmodium berghei NYU-2 from infected mouse blood. Several methods for erythrocyte lysis were investigated, and all were found to yield free parasite preparations variably contaminated by intact parasitized cells. When the necessary precautions were taken to completely remove these contaminant intact erythrocytes, the free parasites were

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not found to possess the ability to invade susceptible erythrocytes *in vitro*.

Even when methods were used which have been reported to yield invasive free parasites, all the infectivity of these preparations could be accounted for by the presence of intact parasitized cells. Therefore, studies were carried out using a method unequivocally known to yield invasive merozoites, i.e. immune lysis *in vivo*. Under these circumstances, it was found that, once freed from the confines of the schizont-infected erythrocyte, the free merozoites rapidly lose invasive ability with time in a non-toxic diluent. This loss of function occurred at 1-2^{o}C, and was exacerbated by washing the parasitized cells prior to sensitization with antibody. It was concluded that the loss of invasive ability with time may be a general property of malarial merozoites and any free merozoites harvested from blood, if invasion is to be studied, must be presented to susceptible host cells within 15 min following their isolation.

Exhaustive efforts were performed to adapt the *in vitro* culture methodology known to support the continuous culture of *P. falciparum* to the *P. berghei* model. Many parameters were varied in these attempts, but none gave better than maintenance of parasite numbers after a single cycle of intracellular development. However, the system employed (RPMI 1640 containing 10% fetal calf serum in an atmosphere of 90%N_2/5%CO_2/5%O_2) did support excellent intracellular development. The limiting factor seemed to be the invasion event. All efforts to continuously culture *P. berghei* over several development cycles failed.

Given the excellent intracellular development seen in the culture trials, and the fact that merozoites were being released by spontaneous schizont rupture, studies were begun to test the immunogenicity of the cultured parasites. Preliminary results of this work indicate that the cultures, if begun with predominantly young ring-stage parasites, may be grown at high parasitemia with excellent development. Reinvasion is minimal, but merozoite release does occur. Using extracts of parasites taken from such cultures at selected times, there appears to be a gradual acquisition of immunogenicity by the parasites with time, which is correlated with increasing numbers of free merozoites. It thus appears that *P. berghei* shares with other malarial parasites the property that the antigens which stimulate functional immunity are merozoite-associated.

Abstract:

Studies were performed to develop a method for the isolation of large numbers of free merozoites of *Plasmodium berghei* NYU-2 from infected mouse blood. Several methods for erythrocyte lysis were investigated, and all were found to yield free parasite preparations variably contaminated by intact parasitized cells. When the necessary precautions were taken to completely remove these contaminant intact erythrocytes, the free parasites were found not to possess the ability to invade susceptible erythrocytes *in vitro*.

Even when methods were used which have been reported to yield invasive free parasites, all the infectivity of these preparations could be accounted for by the presence of intact parasitized cells. Therefore, studies were carried out using a method unequivocally known to yield invasive merozoites, i.e., immune lysis *in vivo*. Under these circumstances, it was found that, once freed from the confines of the schizont-infected erythrocyte, the free merozoites rapidly lose invasive ability with time in a non-toxic diluent. This loss of function occurred at 1-2°C, and was exacerbated by washing the parasitized cells prior to sensitization with antibody. It was concluded that the loss of invasive ability with time may be a general property of malarial merozoites and any free merozoites harvested from blood, if invasion is to be studied, must be presented to susceptible host cells within 15 min following their isolation, even if all procedures are performed in the cold.

Exhaustive efforts were performed to adapt the *in vitro* culture methodology known to support the continuous culture of *P. falciparum* to the *P. berghei* model. Many parameters were varied in these attempts, but none gave better than maintenance of parasite numbers after a single cycle of intracellular development. However, the system employed (RPMI 1640 containing 10% fetal calf serum in an atmosphere of 90% N_2/5% CO_2/5% O_2) did support excellent intracellular development. The limiting factor seemed to be the invasion event. All efforts to continuously culture *P. berghei* over several development cycles failed.

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FOREWORD

This report constitutes a final technical report of work performed under Contract No. DAMD17-75-C-5037, initiated 1 Apr 75. The Contract expires 31 July 1978. The reader is referred to other reports submitted under this Contract, both quarterly and annual, for more information regarding the project.

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee of the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council. The animal facilities are under the continuous supervision of a licensed veterinarian who specializes in laboratory animal care.

Statement of the Problem:

The Contract was awarded for the purpose of pursuing the following objectives:
(1) Detailed evaluation of several methods for the isolation of invasive merozoites of *Plasmodium berghei* from infected mouse erythrocytes. Use of biological, physical, and mechanical means would be tested under direct comparison, and the method best suited for isolation would be chosen if any produced large numbers of viable parasites.
(2) Studies designed to increase the percentage of schizont-infected cells present in the starting cell suspensions were proposed; the primary approach would be the use of preparative density gradient ultracentrifugation.
(3) The percentage of schizont-infected cells present *in vivo* may be increased by inducing synchrony in the donor animals; studies were proposed to attempt synchrony induction, using massive intravenous doses of ring-stage parasitized cells.
(4) Following the determination that free merozoites have a limited extracellular lifespan *in vitro* and the success of other laboratories in cultivating *Plasmodium falciparum* (1,2), the focus of the project shifted to efforts at the adaptation of the published culture methods to the growth of *P. berghei in vitro*.
(5) Parasites harvested from the cultures were evaluated for their functional immunogenicity, i.e. their potential to yield antigens which, when used in a suitable immunization schedule, would induce protection against challenge with viable parasites of the virulent NYU-2 line.

Background:

The successful continuous culture of *P. falciparum in vitro* (1,2) has paved the way for an enormous proliferation of studies designed to permit the speedy development of a vaccine derived from cultured parasites. The culture system as developed by Trager and Jensen (1) requires a stationary layer of cells and modified medium RPMI 1640; using human erythrocytes (outdated from blood banks), near-physiological multiplication rates are attainable. The success of Haynes et al. (2) using a

different medium, indicate that conditions necessary for culture include stationary cells, low O_2 and CO_2 tensions, and a modest parasitemia.

There seems little doubt that the factor limiting prior success in cultivation efforts *in vitro* has been the extreme fragility of the merozoite stage. The fact that both Trager and Jensen (1) and Haynes et al. (2) use stationary cells in their cultures maximizes the chances that an emergent merozoite will in fact encounter a susceptible cell quickly. Assuming the two species are comparable, the reason for haste in permitting merozoite access to red cells appears to be their very rapid loss of invasive ability, first documented with *P. knowlesi* merozoites. This phenomenon has been observed directly (3,4) and also indirectly (5) using the highly synchronous *P. knowlesi*. Since the classical studies of Walter (6) it has been known that *only* the merozoites among a population of free parasites have the capability for invasion. During the course of this project, the P.I. (7) also found *P. berghei* merozoites to have a brief extracellular lifespan, as assessed by their ability to invade susceptible mouse cells. These findings were in contrast to those of Hamburger and Kreier (8,9) and Kreier et al. (10), who concluded their free parasite suspensions following continuous-flow ultrasonic lysis of infected cells--were infective. The P.I. has concluded that the method of Kreier always gives rise to suspensions of cells contaminated with intact parasitized cells which are responsible for such "free parasites"' infectivity (11).

In conjunction with this project, the P.I. has published four articles concerning different aspects of the problem (7,11, 12,13). Additionally, two oral presentations have been given at the annual meetings of the American Society of Tropical Medicine and Hygiene in 1976 (14) and in 1977 (15), and an abstract has been submitted for a paper to be presented at the American Society of Parasitologists conference to be held in November 1978 (16).

Approach to the Problem: Results and Discussion:

The reader is referred to Annual Reports Numbers 3 and 8 for complete summaries of the work performed during the first eighteen months of this project. The final nineteen months of the project, during which time culture trials and immunization experiments were performed, forms the basis of this summary.

The primary medium used in efforts at the successful cultivation of *P. berghei* was modified RPMI 1640, buffered with 25 mM HEPES, in an atmosphere of $90\%N_2/5\%CO_2/5\%O_2$ (1). Numerous modifications of the medium, as well as other experimental variables, have been tested.

The P.I. is aware of the real and postulated differences between rodent and human erythrocytes, but the fact that continuous culture of *P. falciparum* is possible in modified proprietary tissue culture medium was judged to be the logical starting point for efforts at culture of rodent parasites. The culture trial results were largely negative; data will

thus be summarized briefly.

Experiments have been done using P. chabaudi (cloned), P. falciparum FVO strain, P. falciparum Camp., and P. berghei NYU-2. The rodent parasites are studied using infected blood collected using cardiac puncture. Cells are washed using centrifugation in culture medium without serum. The final wash and resuspension are done in complete medium supplemented with serum (usually fetal calf serum from GIBCO). Normal erythrocytes are admixed with parasitized cells to lower the parasitemia to <2% when necessary, and the buffy coats are typically removed to lower the leucocyte content of the starting cell suspensions.

Initial experiments were done in an atmosphere of 5% CO_2. Subsequently, candle jars were evaluated (1), and currently the defined mixture mentioned above is used. Stationary cultures are inoculated in 35 x 10 mm petri plates, and the red cell concentration in starting cultures is typically adjusted to ca. 5×10^8 cells/ml. Incubation is at 37°C, and medium changes are performed at various intervals dependent on the source of red cells and parasites [for mouse cells, sampling times are typically 12, 18, and 24 hr; for rat cells, 18, 24, and 30 hr are normally chosen]. Under the conditions employed, reinvasion rates are very low and the results were found to be predictable if the cultures were only monitored for a single cycle of development. However, efforts were made to keep the parasites alive over several cycles, in spite of their decreasing numbers.

The medium employed typically gives the following results when P. berghei is cultured in rat erythrocytes:

time	ring	trophozoite	schizont	par/10,000rbc	MR
0 h	40	59	<1	141	--
24 h	38	40	22	47	0.33

If the aspirated medium taken during medium changes is examined, there is a great deal of merozoite release occurring in this medium. But the parasites show only modest penetration, and 20-80% of the parasites are usually lost after one developmental cycle; only occasionally may even maintenance be achieved. Notice that after 24 hr, there are numerous schizonts present, suggesting that the developmental cycle in vitro may be longer than the 24 hr cycle this parasite normally shows in the rat host. Intracellular development appears normal, at least with respect to morphology of all stages. Invasion is not precluded in this medium, but the rate is very low.

Numerous approaches were taken in efforts to improve the reinvasion rate while simultaneously preserving intracellular morphology. Consistently better results are achieved when rat cells are used for cultures than when mouse cells are employed; this is likely due to greater numbers of merozoites being produced in rat cells per schizont.

The following variables were tested in the culture trials, in efforts to increase the multiplication rate:

Host species (rat, mouse)
Parasite species (*P. berghei*, *P. chabaudi*)
Mechanical factors
Serum: source
absorption
concentration
Medium: formulation
supplementation
dilution
osmolarity
pH
buffer concentration
gas phase
± antibiotics
change frequency
Erythrocytes: storage 4^oC
pre-incubation 37^o (receptor lability)
processing temperature
anticoagulant
leucocyte/platelet depletion
concentration/parasitemia
Host: age
infection age
Anesthesia toxicity
Incubation temperature

All the variables mentioned above were found not to give appreciable improvements in the multiplication rate over ranges deemed physiological by the P.I. Whatever is limiting the cultures appears associated with the schizogonic event(s), producing either altered merozoites incapable of significant invasion, or else causing some inhibition of the invasion process itself. There are an almost infinite number of variables which could be responsible for the different behavior observed with the rodent parasites as compared to *P. falciparum*; but it must be emphasized that *P. falciparum* produces many more merozoites per schizont than does *P. berghei*, and this difference may be a major reason for the results obtained.

In the course of the culture trials, it became obvious that with frequent medium changes and moderate erythrocyte concentrations (10^8/ml), parasite morphology and development may be preserved throughout the first 24 hr, even if mouse erythrocytes are employed and relatively high starting parasitemias (20%) are present in the starting cells. It was thus reasoned that although reinvasion is minimal, the fact that the parasites do undergo development to the point of schizont rupture should permit a study of cultured merozoites' antigenicity. Since it is well known that the merozoites of *P. knowlesi* (17) can stimulate a broad-spectrum variant-transcending immunity in the rhesus, the possibility that merozoites from the *P. berghei* cultures may be immunogenic seemed worth pursuing.

This hypothesis seemed especially provocative since the antigens associated with the merozoite may be the only antigens capable of triggering functional immunity. The successful immunization experiments of Murphy and Lefford (18) showed that immunization against *P. berghei* NYU-2 is possible with dead antigen derived from peripheral blood. A preliminary experiment was thus performed to explore the cultured parasites' immunogenicity.

Hybrid B_6D_2 F_1 mice were randomized and divided into 8 groups of 10 animals per group. Washed parasitized cells from 35 hybrid donors were inoculated into 500 ml culture flasks (100 ml/flask) in RPMI 1640 (1) containing 10% fetal calf serum. The parasitized cells were ring-stage enriched by aspiration of the uppermost layer of brown cells from the packed cell column following each centrifugation during washing. The starting cell suspension contained 20% parasitized cells, of which 82% were rings, 18% were trophozoites, and <1% were schizonts. All flasks were gassed and tighly sealed. Incubation was performed in a shaking water bath at 37°C with gentle shaking, and flasks were removed from the bath at times 0, 5, 10, 15, and 20 hr. All flasks were held at 4°C until the last sampling time, and then cells were washed/lysed for free parasite antigen according to Murphy and Lefford (18). Protein determinations were performed and all antigen preparations were normalized by dilution to a concentration of 949 μg/ml. In addition to the time-course flasks, additional flasks were prepared containing mixed infected cells (not ring-enriched) and alternatively brown layer cells aspirated from the top of the packed cell column during washing. These were processed in a fashion identical to that described for the 0 hr sample.

Mice were immunized intravenously using 0.3 ml formolized free parasite antigen containing 285 μg protein. The antigen was inadvertently suspended in 0.9 M NaCl, and there was consequent mortality due to osmotic shock on injection. This oversight was not recognized until most of the animals had been injected, and thus the number of animals in each group which survived the osmotic shock was variable:

Group	Number (surviving injection with antigen hypertonic	Differential count* ring	troph	schizont	free mz
brown cells	7	29	67	4	--
mixed inf cells	3	77	22	1	--
0 hr	9	82	18	<1	--
5 hr	7	51	46	3	--
10 hr	9	52	44	3	+
15 hr	10	58	37	4	+++
20 hr	10	58	40	2	+++
unimmunized controls	11	na	na	na	na

*The differential counts above represent percentages of each stage present at the time the cells were processed for antigen.

All mice were challenged 2 weeks post-immunization with 5×10^2 parasitized cells/g with viable parasites. Thin blood films were prepared daily until all animals had succumbed to the infection. Results of this experiment are shown in Figs. 1 & 2.

The unfortunate accident involving the hypertonic saline renders this experiment preliminary, but still useful to reveal whether the idea is worthy of further pursuit. All mice died due to the infection, but the trend is obvious. There was a progressive tendency towards prolonged survival time (Fig. 1) with increasing time of incubation in vitro prior to antigen preparation.

The hybrid mouse employed typically responds to the NYU-2 line of P. berghei by showing an initial early peak of parasitemia, during which time there is some mortality, followed by a protracted course of infection which ultimately leads to 100% mortality. As may be seen in Fig. 2, the animals immunized with cultured parasites experienced a depression in the first peak of parasitemia, and the general trend was as that exhibited by the mortality data in Fig. 1--longer incubation times in vitro led to more potent depressions in the first peak of parasitemia following challenge.

The differential parasite counts of the cultured cells were fairly stable, in terms of the percentages of each stage present. This is to be expected, since the medium does support invasion at a low frequency, and the fact that the parasites stay at the ring stage for greater than half the intracellular cycle in vivo. The major difference between the antigen preparations was thus the number of free merozoites present, and these were numerous in the 15 and 20 hr cultures. Since all mice were immunized with equivalent amounts of protein, the results indicate that the free merozoites contain antigen of superior quality for stimulating functional immunity, rather than simply being quantitatively superior to intracellular stage-antigen. An alternative explanation is that there are materials associated with the early stages which interfere with the induction of immunity. This is a possibility, as the 0 hr cells contained many more total rings than did the 20 hr samples, as well as containing a slightly higher percentage of rings.

Conclusions and Recommendations:

This study has provided significant information regarding the merozoites of Plasmodium berghei. This delicate extracellular stage may best be thought of in terms of a stage specialized for the transport of the parasite's genetic material between erythrocytes. The transit between cells is one which is filled with potential hazards, including the presence in many cases of host antibody known (in P. knowlesi) to profoundly affect the merozoite stage. The merozoites of P. berghei are quite fragile, and lose invasive ability within a few minutes after their release from the confines of the schizont-infected cell.

Furthermore, this property appears to be true if the merozoites are isolated in vitro but permitted to invade under the best of circumstances, i.e. in the vascular system of the mouse (7).

Although great strides have been made in malariology in the past few years, especially with respect to the culture in vitro of P. falciparum (1,2), there is an enormous amount of basic information regarding merozoite-erythrocyte interactions and merozoite antigens which needs to be elucidated prior to the successful development of a vaccine which will have benefit for our armed forces. There is a strong temptation to channel all efforts into studies on P. falciparum and push for the vaccine development now, rather than taking the more judicious and time consuming approach of continuing to support basic studies on other models. Given the tight nature of USAMRDC's available funds for research, this is understandable. But the power of malarial parasites to outwit the best efforts at control should not be underestimated. It is hoped that funds will continue to be available for studies on rodent and avian malarial parasites, as these models present ideal avenues for answering many extant and important questions about malaria.

The P.I. wishes to express his gratitude to the Command for its continued support of this research over the past three years.

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Figure 1--Mortality Data, Time-Course Experiment

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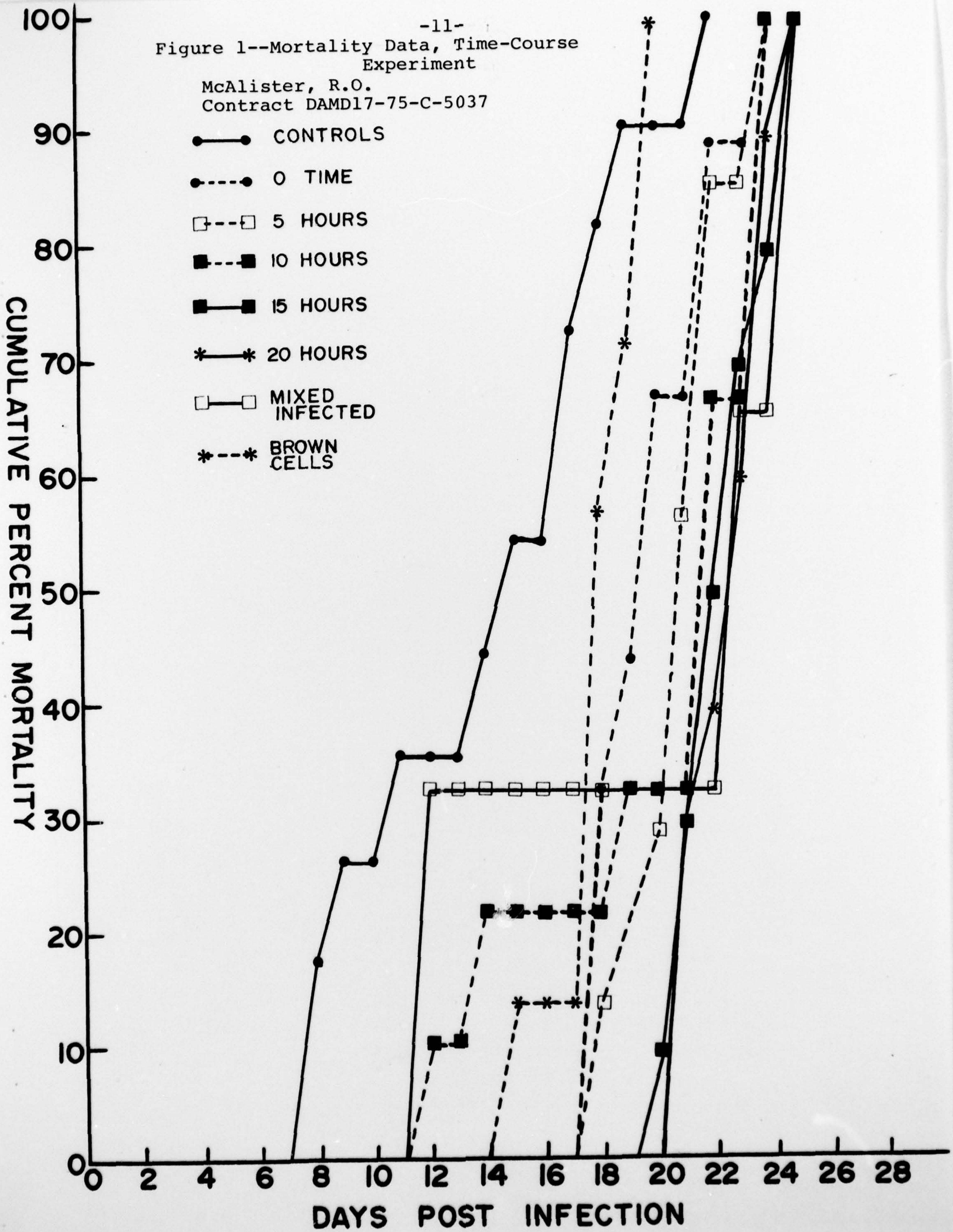

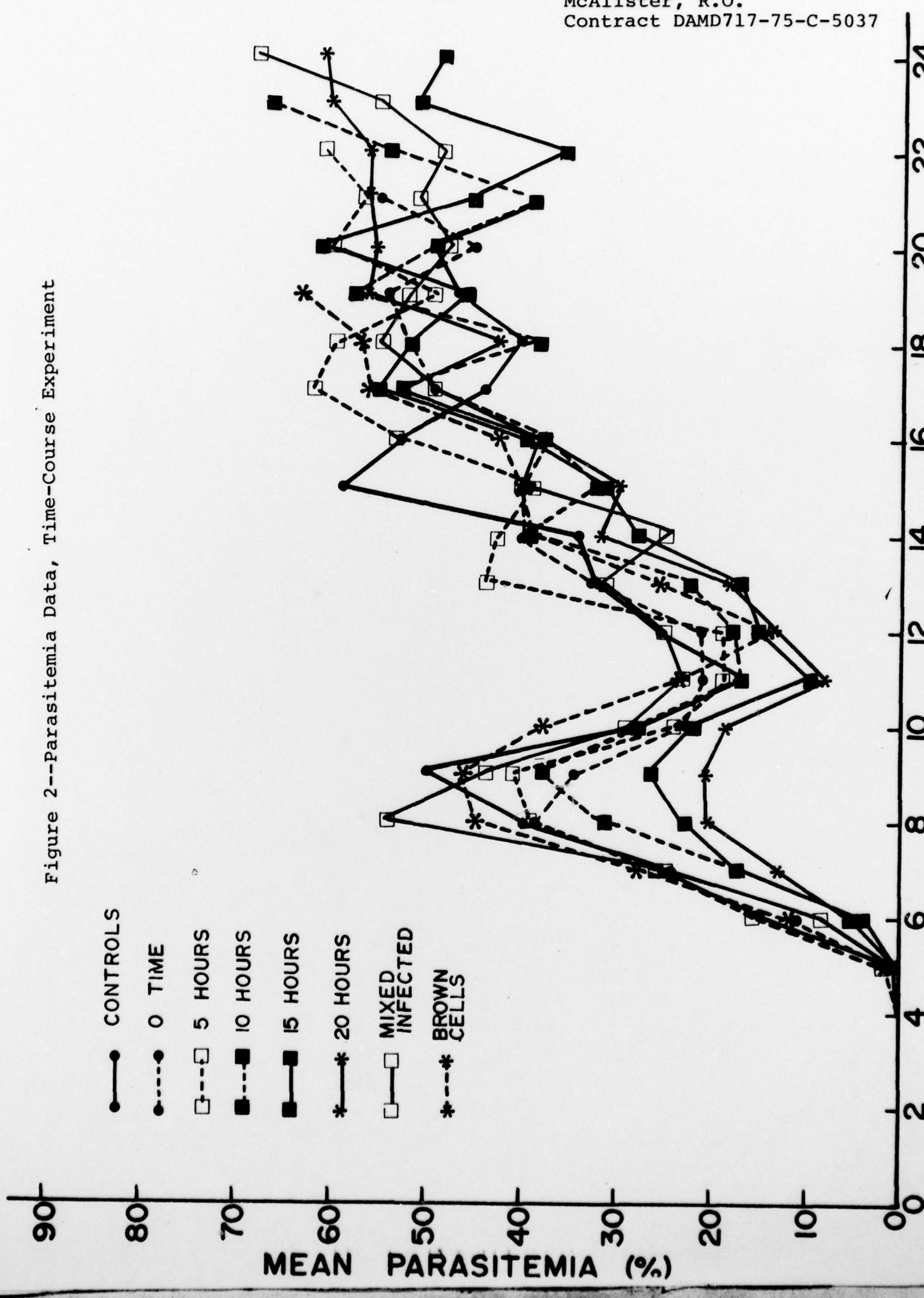

Figure 2--Parasitemia Data, Time-Course Experiment